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IDENTIFICATION OF ORIGINAL "KHALAS" CULTIVAR DATE PALM BY USING OF ELECTROPHORETIC ANALYSIS OF ISOENZYMES

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ABSTRACT

Date palm can be grown from seeds or offshoots. "Khalas" cultivar trees in Al-Ahsa oasis differed in their dates quality. Identification of the original "Khalas" cultivar is important to guarantee the superior characteristics of cultivated cultivar trees. In this research isoenzymes extracted from different date palms leaflets of "Khalas" cultivar, which were collected from 10 different locations of Al-Ahsa to determine how uniform the trees were. Extracted isoenzymes have been analyzed by PAGE electrophoresis. The results showed polymorphism in EST and GOT isoenzymes patterns, the number of EST bands ranged between 2-7 bands, while ranged in GOT between 2-4 bands. Accordingly the differences of isoenzymes electrophoretic patterns were revealed by UPGMA clustering method. The results collectively revealed genetic variations of the cultivar trees, but could not represent collectively any proposed original "khalas" cultivar trees. However, identification of the original cultivar is important to guaranty the superior characteristics of cultivated cultivar trees, More genetic and biochemical studies should be conducted to support this conclusion.

KEYWORDS: Date Palm, Electrophoresis, Isoenzymes, Genetic Variations, UPGMA

INTRODUCTION

Date palm (*Phoenix dactylifera* L., 2n=2x=36) is a monocotyledonous and dioeciously species belonging to Arecaceae family. It includes 225 genus and 2600 species (Corner, 1966), and widely cultivated in arid regions of the Middle East and North Africa, (Hamza et al., 2011, Elshibli, 2010, Al-Yahyai and Al-Khanjari, 2008, Ould Mohammad et al., 2007, Azeqour et al., 2002, El-Tarras et al., 2007, Elmeer et al., 2011). It is widely distributed in the Eastern Province of Kingdom of Saudi Arabia. There are more than 70 cultivars that have been grown there for ages (Asif et al., 1982; Al-Ghamdi and Al-Kahtani, 1993). Date palm can be propagated by seeds, which usually produce trees bearing inferior fruits. Offshoots are more preferred for conventional propagation because they produce true-to-type trees with fruit quality identical to the mother tree (Asif et al., 1982; Al-Ghamdi and Al-Kahtani, 1993).

The high stability of protein profile makes protein electrophoresis a powerful tool in elucidating the origin and the evolution of cultivated plants (Ladizinsky and Hymowitz,1979), as isozymic analysis has proved useful to identify differences in gene expression in various organs of the same plant or to differentiate between closely related cultivars (Ben-Hayyim et al., 1982), Therefore, isoenzyme pattern analysis by gel electrophoresis has been used in higher plants to study various problems in breeding, genetics, taxonomy, physiology, (Al-Helal, 1988, 1992, 1994, Barta et al., 2003, El-Sherabasy et al., 2008, Zivdar et al., 2008, Khan et al., 2009, Gonzalez et al., 2004, Petrova et al., 2006, Ould Mohammad et al., 2001, Saker et al., 2002, Al-Fredan, 2013, Pathak, 2011) as it has been appreciated as a biochemical tool for studying the phylogentic relationships (Al-Yahyai and Al-Khanjari, 2008).The phylogenetic analysis were used to study the genetic relatedness between and among cultivars (Elham et al., 2010; Munshi and Osman, 2010; El Akkad, 2004;

Haider et al., 2012, Khan et al., 2009). So, it would be helpful in recognizing the originals cultivars. On the other hand, "Khalas" cultivar is one of the most economical date palm cultivars grown in Al-Ahsa oasis at the eastern province of Saudi Arabia.

It is known locally that "Khalas" Cultivar trees are differed in their fruit quality depending on the location even within the oasis (Al-Mulla, 1979; Asif et al., 1982). AlIssa et al. (2006) showed in their comparative study to genetic and biochemical differences within "Khalas" cultivar trees growing in two localities, as were shown in other study to protein patterns differences within "Khalas" cultivar trees from different locations of Al-Ahsa oasis (AlIssa, 2013). The main aim of the present study is to analyze whether their genetic diversity among "Khalas" cultivar trees according to ten locations of Al-Ahsa oasis using isoenzymes profiling. Consequently, 'Khalas" cultivar have been selected for electrophoretic isoenzymes analysis by polyacrylamide gel electrophoresis (PAGE), leaflet tissues collected from 10 different locations within Al-Ahsa oasis which considered as the origin of this cultivar.

MATERIALS AND METHODS

The samples were collected from juvenile green leaflets from 30 years old and above "Khalas" cultivar trees in each location, the area were samples collected covered the most of Al-Ahsa oasis areas for date palm cultivation, which divided in to three areas: North, Middle and East villages (Table 1, Figure 1). The trees were offshoot of previous mother trees which were grown in the same location. The samples transferred immediately to liquid nitrogen, then to deep freezer -20 ° C until the time of usage. The samples were cut into 2x2 mm before ground in mortar with liquid nitrogen prior to be subjected to Isoenzyme electrophoretic analysis using the PAGE technique.

Isoenzymes Extraction: The samples were ground in a mortar by liquid nitrogen, then the Isoenzymes extracted by homogenizing 100 mg of ground leaflet samples by half volume stainless steel beads with 100 µl extracting buffer (0.075 M Sodium Phosphate dehydrogenase pH 7.25. (1.17 gm/100 ml DIW) + 10% glycerol.+ 4% PVP) at 9/4 min., followed by 10/2 min. by using of (Bullet Blender Homogenizer), then incubated overnight at 4° C. The crude extract was vortexed by using of (VELP vortex mixer), then centrifuged at 12500 rpm for 10 min by using of (Eppendorf centrifuge 5424), the supernatant was moved to new tubes, while discarded the debris.

Isoenzymes Concentration: To the supernatant 1 ml of chilled acetone was added, vortexed, centrifuged at 12500 rpm for 10 min., then acetone discarded, this step repeated twice. After the acetone have been discarded the precipitated Isoenzymes was incubated at room temperature for 15 min's

Resuspending Isoenzymes: The Isoenzyme are redissolving in 60 µl. of extracting buffer, then vortexed, centrifuged at 12500 rpm for 10 min, after that loaded in 30 µl.

Electrophoresis: Discontinuous vertical Polyacrylamide gel electrophoresis (PAGE) was carried out in 7.5% gels according to Davis (1963) and Ornstein (1964) procedures with some modifications, in 12.5% resolving gels (2.25 gm Acrylamide - bis acrylamide 0.05 gm + 11.25 ml (1.5 M tris/HCl buffer pH 8.8) + 17 ml DW + freshly prepared 0.12 ml (10% amm. persulphate) + 0.008 ml TEMED] and stacking gels (0.45 gm Acrylamide - bis acrylamide 0.0125 gm + 1.25 ml (0.5 M tris/HCl buffer pH 6.8) + 8.5 ml DW + freshly prepared 0.025 ml (10% amm. persulphate) + 0.005 ml TEMED]. The run buffer was prepared by dissolving 3.0 gm Tris + 14.1 gm Glycine in DW to 1 L, while the pH adjusted to 8.3, and the electrophoresis was carried out by using of (BioRad, Broka 0.75 mm) mini electrophoresis system with (BioRad PowerPac Basic) at 100-150 Volts.

Assay of Enzymes: (GOT): Gels stained for Glutamate Oxaloacetate Transaminase (GOT, EC. 2.6.1.1) were incubated in (100 ml) L-aspartic acid (500 mg) + Ketoglutaric acid (70 mg) + Pyridoxal 5-phosphate (10 mg) + Fast blue B salt (200 mg) + 0.2 M sodium acetate butter pH 5.0. (EST): Gels stained for Non-specific esterase (EST, EC. 3.3.1.2) were incubated in (90 ml) α-naphthyl acetate (15 mg) + Fast blue B salt (90 mg) + 0.2 M sodium acetate butter pH 5.0. (GDH) Gels stained for Glutamate dehydrogenase (GDH, EC. 1.4.1.2) were incubated in (75 ml) NAD (40 mg) + Phenazine methosulphate (2 mg) + Nitroblue tetrazolium (35 mg) + Sodium glutamate (300 mg) + 0.1 M tris\HCl buffer pH 9.0. (SOD) Gels stained for Superoxide Dismutase (SOD, EC. 1.15.1.5) were incubated in (75 ml) NAD (40 mg) + Phenazine methosulphate (2 mg) + Nitroblue tetrazolium (35 mg) + 0.1 M tris\HCl buffer pH 9.0. (LAP) Gels stained for Leucine-aminopeptidase (LAP, EC. 3.4.1.1) were incubated in (30 ml) L-leucyl- β – naphthylamide (8mg/ml H2O) (3 ml) + 0.85% (w/v) NaCl (24 ml) + 0.2 M KCN (3 ml) + Fast blue B salt (60 mg) + 0.2 M sodium acetate butter pH 5.0. Gels were destaind in a solution of 3:1:6 proportional of ethanol, acetic acid, distilled water respectively.

Band Scoring and Analysis: Isoenzyme bands have been scored, only unambiguous bands coded for presence / absence (I/O). Quantitative evaluations of the Isoenzyme bands have been done by eye, as the following:

Easy visualized	▬	Visualized	—	Hard visualized	.....

Data Analysis: The results obtained from Isoenzyme patterns were analyzed statistically, while relative movement of each Isoenzyme band was determined (figure 2), Isoenzyme bands were scored depending on their presence (I) or absence (O) (table 2). Jaccard`s distances was determined (table 3), and hierarchical clustering was constructed by Unweighted pair group method with arithmetic average [UPGMA] by using MEGA4 software (Tamura, et al, 2011).

Table 1: Locations and Distributions of "Khalas" Cultivar Date Palms with Corresponding Abbreviations

Area	North				Middle			East		
No.	1	2	3	4	5	6	7	8	9	10
Location	SH[a]	G[b]	J[c]	M[d]	OY[e]	AM[f]	B[g]	A[h]	OM[i]	T[j]

a. Al-Shoa`bah, b. Al-Gurain, c. Julaijalah, d. Al-Mutairfi, e. Al-Oyuni, f. Ain Merjan, g. Al-Battalia, h. Al-Aqar, i. Al-Omran, j. Al-Taraf.

Figure 1: Map of Al-Ahsa Oasis were Cultivar Samples Locations Mentioned by Abbreviations

RESULTS

In the present study, the isoenzymes extracts of different date palm trees "Khalas" cultivar leaflets samples collected from ten locations were subjected to PAGE analysis. GDH, LAP and SOD isoenzymes did not show enzymatic

activity as they have not developed any electrophoretic bands. EST isoenzymes were developed seven bands in tow zones, the 1st zone, ranged between 0.371-0.621 relative movments, which characterized by low activity bands, while the 2nd zone ranged between 0.69-0.787 relative movments (Figure 2, Table 2). EST bands were ranged between 2 bands in M location and 7 bands in OM and J locations, with polymorphism ratio ranged between 50% in M location to 86% in OM and J locations, while there is only one EST polymorphic band (rf=0.787) among all locations trees, while the polymorphic bands ranged between one band in M location and six bands in OM & J locations (table 3). The Genetic Distances based on Jaccard's distances method on the basis of presence and absence of EST isoenzymes bands (Table 2), ranged between 0.143-0.750 (Table 4), The minimum variation values according to EST iosenzymes of 0.143 was observed in between J and A locations samples, while the maximum variation values of 0.750 was observed between T and M, locations samples. Different isoenzyme patterns were revealed by UPGMA clustering method based on EST isoenzymes. EST isoenzymes dendrogram revealed different clusters according to UPGMA dendrogram, 1st. cluster composed of OY, AM, T, G, J and A locations samples, 2nd cluster included OM, SH and B locations samples, 3rd cluster included only M location samples. (figures 3)

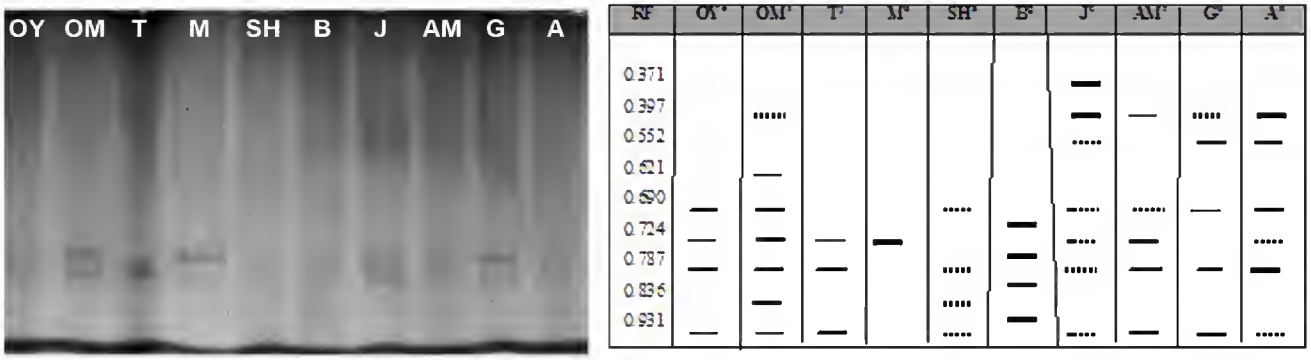

Figure 2: Electrophoretic Zymogram Patterns of EST Isoenzymes in Khalas Cultivar from Different Locations

Table 2: The Bands of EST Isoenzymes, Represented by O = Absent, I = Present

RF	OY^e	OM^i	T^j	M^d	SH^a	B^g	J^c	AM^f	G^b	A^h
0.371	O	O	O	O	O	O	I	O	O	O
0.397	O	I	O	O	O	O	I	I	I	I
0.552	O	O	O	O	O	O	I	O	I	I
0.621	O	I	O	O	O	O	O	O	O	O
0.690	I	I	O	I	I	I	I	I	I	I
0.724	I	I	I	O	O	I	I	I	O	I
0.787	I	I	I	I	I	I	I	I	I	I
0.836	O	I	O	O	I	I	O	O	O	O
0.931	I	I	I	O	I	O	I	I	I	I
Total	4	7	3	2	4	4	7	5	5	6

Table 3: Monomorphic and Polymorphic Bands of EST Isoenzymes

Isoenzymes	Total Bands	Monomorphic	Polymorphic	(%)
OY^e	4	1	3	75
OM^i	7	1	6	86
T^j	3	1	2	67
M^d	2	1	1	50
SH^a	4	1	3	75
B^g	4	1	3	75
J^c	7	1	6	86
AM^f	5	1	4	80

Table 3: Contd.,				
G^b	5	1	4	80
A^h	6	1	5	83

Table 4: The Data Matrix of Jaccard`s Distance Analysis of EST Isoenzymes within Different Samples

Locations	OY^e	OM^i	T^j	M^d	SH^a	B^g	J^c	AM^f	G^b	A^h
OY^e	0.000									
OM^i	0.429	0.000								
T^j	0.250	0.625	0.000							
M^d	0.500	0.714	0.750	0.000						
SH^a	0.400	0.429	0.600	0.500	0.000					
B^g	0.400	0.429	0.600	0.500	0.400	0.000				
J^c	0.429	0.444	0.571	0.714	0.625	0.625	0.000			
AM^f	0.200	0.286	0.400	0.600	0.500	0.500	0.286	0.000		
G^b	0.500	0.500	0.667	0.600	0.500	0.714	0.286	0.333	0.000	
A^h	0.333	0.375	0.500	0.667	0.571	0.571	0.143	0.167	0.167	0.000

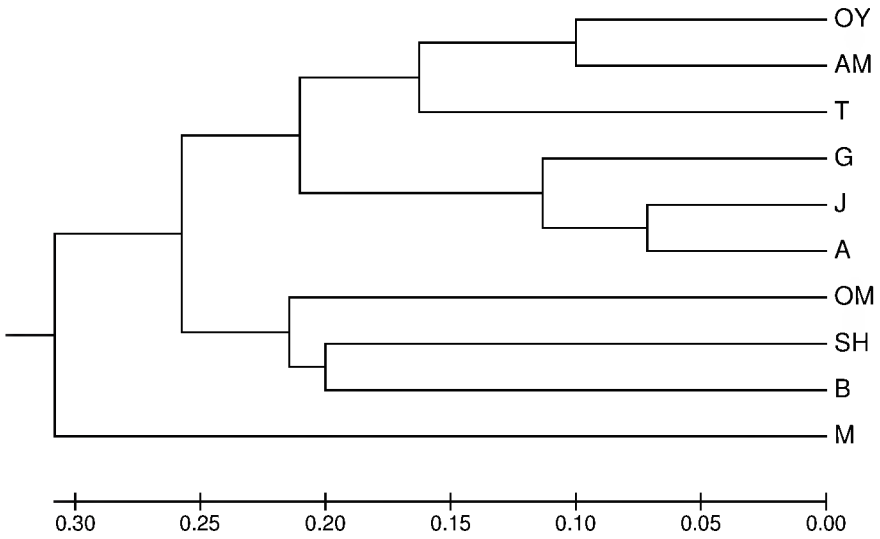

Figure 3: UPGMA Based Dendrogram According to EST Isoenzymes Electrophoretic Patterns Based on Jaccard's Distances, Showing the Relationships of "Khalas" Cultivar among the Different Locations Samples

GOT isoenzymes were developed four bands in tow zones, the 1st zone, ranged between 0.12 and 0. 21 relative movements, while the 2nd zone ranged between 0.54 and 0.74 relative movements, all GOT isoenzymes bands characterized by low activity (Figure 4, Table 5). GOT bands ranged between 2 bands in OY, M, B, J, G and A locations and 4 bands in OM and T locations, with polymorphism ratio ranged between 0% in OY, M, B, J, G and A locations to 50% in OM and T locations (Table. 6) , There is tow GOT monomorphic bands (rf=0.12 & rf=0.54) among all locations trees, while the polymorphic bands ranged between one band in SH & AM locations and tow bands in OM & T locations (table. 6).

The Genetic Distances based on Jaccard's distances method on the basis of presence and absence of GOT isoenzymes bands (Table 5), ranged between 0.00-0. 500 (Table 7), The minimum variation values according to GOT iosenzymes of 0.00 was observed in between OY, M, B, J, G and A locations samples, and OM and T locations samples, while the maximum variation values of 0.50 was observed between OY, M, B, J, G and A with OM and T locations samples. GOT isoenzymes dendrogram revealed tow clusters according to UPGMA dendrogram, 1st. cluster composed of

G, A, J, B, M, and OY locations samples, 2nd cluster included OM, T, SH and AM locations samples (figures 7).

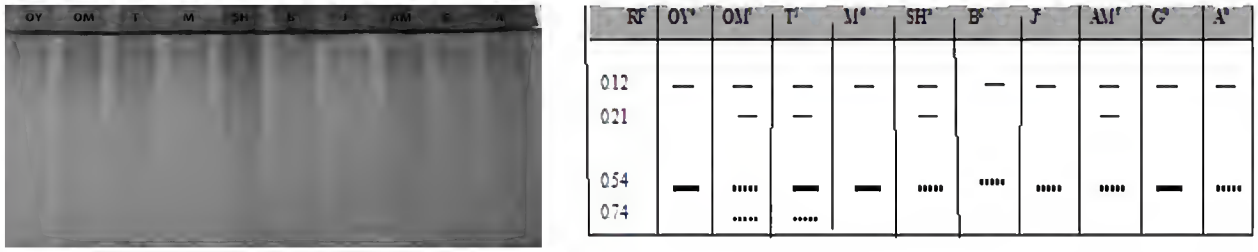

Figure 4: Electrophoretic Zymogram Patterns of GOT Isoenzymes in Khalas Cultivar from Different Locations

Table 5: The Bands of GOT Isoenzyme, Represented by O = Absent, I = Present

Isoenzymes	Total Bands	Monomorphic	Polymorphic	(%)
OY^e	2	2	0	0
OM^i	4	2	2	50
T^j	4	2	2	50
M^d	2	2	0	0
SH^a	3	2	1	33
B^g	2	2	0	0
J^c	2	2	0	0
AM^f	3	2	1	33
G^b	2	2	0	0
A^h	2	2	0	0

Table 6: Monomorphic and Polymorphic Bands of GOT Isozymes

RF	OY^e	OM^i	T^j	M^d	SH^a	B^g	J^c	AM^f	G^b	A^h
0.12	I	I	I	I	I	I	I	I	I	I
0.21	O	I	I	O	I	O	O	I	O	O
0.54	I	I	I	I	I	I	I	I	I	I
0.74	O	I	I	O	O	O	O	O	O	O
Total	2	4	4	2	3	2	2	3	2	2

Table 7: The Data Matrix of Jaccard`s Distance Analysis of GOT Isoenzymes within Different Samples

Locations	OY^e	OM^i	T^j	M^d	SH^a	B^g	J^c	AM^f	G^b	A^h
OY^e	0.000									
OM^i	0.500	0.000								
T^j	0.500	0.000	0.000							
M^d	0.000	0.500	0.500	0.000						
SH^a	0.333	0.250	0.250	0.333	0.000					
B^g	0.000	0.500	0.500	0.000	0.333	0.000				
J^c	0.000	0.500	0.500	0.000	0.333	0.000	0.000			
AM^f	0.333	0.250	0.250	0.333	0.000	0.333	0.333	0.000		
G^b	0.000	0.500	0.500	0.000	0.333	0.000	0.000	0.333	0.000	
A^h	0.000	0.500	0.500	0.000	0.333	0.000	0.000	0.333	0.000	0.000

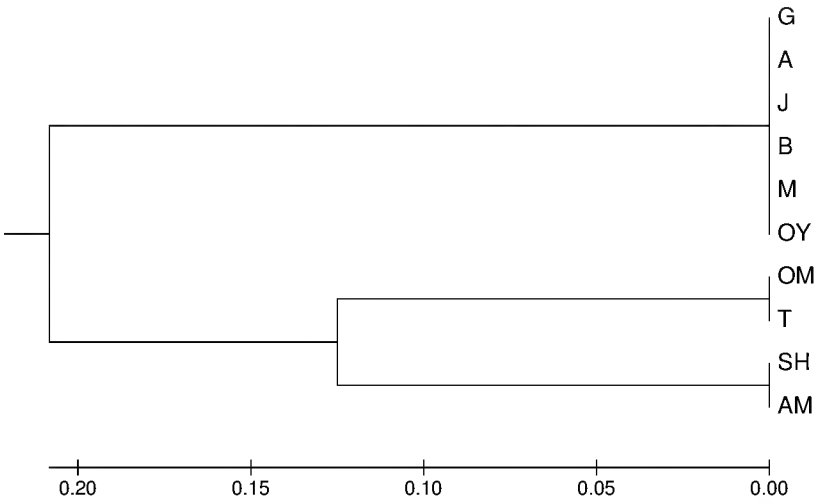

Figure 5: UPGMA Based Dendrogram According to GOT Isoenzymes Electrophoretic Patterns Based on Jaccard's Distances, Showing the Relationships of "Khalas" Cultivar among the Different Locations Samples

DISCUSSIONS

Extract preparation of date palm tissues was very difficult due to tissue toughness and the presences of abundant secondary metabolites, therefore the protein concentration of such extracts going to be low and the their results of electrophoretic analysis accordingly sometimes irreproducible, this is what happen in this work. Similar studies have not been available to be discussed with these results, because it is known that the cultivars propagated by offshoots, which produce plants identical to the mother plant, therefore it`s distracted by researchers from comparison studies of trees belonging to the same cultivar. This study have been done to recognize and identify the original "Khalas" cultivar trees among many "Khalas" cultivar trees growing in Al-Ahsa oasis, since genetic differences have been recorded within "Khalas" cultivar trees in tow locations (Al-Issa, 2006) and within ten locations in Al-Ahsa oasis, based on protein pattern analysis (Al-Issa, 2013). It has been conducted by this study on "Khalas" cultivar trees that there is differences in EST and GOT isoenzymes patterns.

This observation is in concordance with the previous study conducted by (Al-Issa, 2006). The same results of dissimilarity within the same cultivar trees were recorded on flowering dogwood trees (*Cornus florida* L.) which interpreted by mislabeling of the original samples (Naomi, *et al.*, 2007). The electrophoretic patterns of EST isoenzymes were not agreed with the electrophoretic patterns of GOT, since EST electrophoretic patterns revealed wide range of polymorphism in comparison with GOT isoenzymes (Tables. 3 and 5), Due to the wide range of polymorphism EST isoenzymes could not represent any proposed original "khalas" cultivar trees, while the GOT isoenzymes of OY, M, B, J, G and A locations samples revealed monomorphism, it could be suggested as representative for the original "khalas" cultivar trees, but this suggestion opposed with the EST results, and with the reality of these GOT isoenzymes bands were few in number and poor in quality (Figure 3). These results were not in concordance with previous study (Al-Issa, 2013), which were suggested a cluster of monomorphic samples of (OY, OM, T and SH locations) as an original "Khalas" cultivar trees, since these locations trees have been scattered in the present study as revealed in the dendrogram of UPGMA clustering method (Figure 3 and 5).

These results could not represent collectively any proposed original "khalas" cultivar trees, but may support the conclusion conducted by the previous study (Al-Issa, 2013) of (OY, OM, T and SH locations samples) taking in the

cosideration the exception of OY location tree. These results support the probability of some cultivar trees grown from seeds, were resembled the original ones, then distributed later as an original cultivar (Devand and Chao, 2003). So, it could be concluded from this study that there is a genetic differences among "Khalas" cultivar date palms, and the Identification of the original "Khalas" cultivar is important to guaranty the superior characteristics of cultivated trees. Undoubtedly it needs to be subject to more studies. Furthermore, DNA markers should be used to show the genetic structures and variations within "Khalas" cultivar trees, to confirm the fingerprint of the original "Khalas" cultivar. In the long term, the goals should be included the recognizing of the other original cultivars of date palm trees which represent nutritional values and economical importance.

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